

# Nucleic acid aptamers as antithrombotic agents: Opportunities in extracellular therapeutics

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## Summary

Antithrombotic therapy for the acute management of thrombotic disorders has been stimulated and guided actively by our current understanding of platelet biology, coagulation proteases, and vascular science. A translatable platform for coagulation, based soundly on biochemistry, enzymology and cellular events on platelets and tissue factor-bearing cells, introduces fundamental constructs, mechanistic clarity, and an unparalleled opportunity for accelerating the development and

clinical investigation of both disease- and patient-specific therapies. In the current review, we build upon and expand substantially our observations surrounding nucleic acids as antithrombotic agents.

## Keywords

Nucleic acids, RNA and DNA aptamers, protein-binding oligonucleotides, anticoagulant therapy

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## Introduction

Antithrombotic therapy for the acute management of thrombotic disorders has been stimulated and guided actively by the fields current understanding of platelet biology, coagulation proteases, and vascular science. A translatable platform for coagulation, based soundly on biochemistry, enzymology and cellular events on platelets and tissue factor-bearing cells, introduces fundamental constructs, mechanistic clarity, and an unparalleled opportunity for accelerating the development and clinical investigation of both disease- and patient-specific therapies.

In the current review, we will build upon and expand our observations surrounding nucleic acids as antithrombotic agents summarised previously in *Thrombosis and Haemostasis* (1).

## Distinguishing characteristics

Protein-binding oligonucleotides (aptamers), as described previously (1) are single-stranded nucleic acids that inhibit a selected target protein or small molecule's function by folding into a specific three-dimensional structure which permits high affinity binding. For the purpose of this review, we will focus on protein-binding aptamers which, by design, impart antithrombotic effects (► Table 1).

## Technology and design

*In vitro* systematic evolution or selection of ligands by an exponential enrichment allows the identification of oligonucleotides showing properties of interest (2). Unlike functional nucleic acids, including antisense oligonucleotides, ribozymes or siRNAs, our current generation of aptamers do not exert their effects intracellularly (3).

The initiating substrate for aptamer generation is a combinatorial (shape) library composed of single-stranded nucleic acids (RNA, DNA, or modified RNA), typically containing 20 to 40 randomised positions ( $10^{24}$  different sequence possibilities for library containing a 40 nucleotide random region) (1). To achieve isolation of high-affinity nucleic acid ligands, an initial purification step, technically referred to as SELEX (Systematic Evolution of Ligands by EXponential enrichment), is employed (► Fig. 1) (4–5).

Prior to evaluation of aptamers as potential therapeutics or other *in vivo*-uses (e.g. target validation), several post-selection optimisation steps are typically performed, include minimising aptamer length, achieving stability in biological systems by substituting ribonucleotides with 2'-amino, 2'-O-alkyl or 2'-fluoro-nucleotides and protection of exonuclease digestion through 3' end capping, and attenuating renal clearance by conjugation of a polyethylene glycol (PEG) carrier. Based on the needs and specifications required for a given indication, an aptamer can be designed to have a half-life ranging from several minutes to greater than 100 hours (h) (1).

**Table 1: Nucleic acid aptamers as antithrombotic agents.** SELEX (Systematic Evolution of Ligands by Exponential Enrichment); f (factor); TF (tissue factor); VWF (von Willebrand Factor); CAD (coronary artery disease).

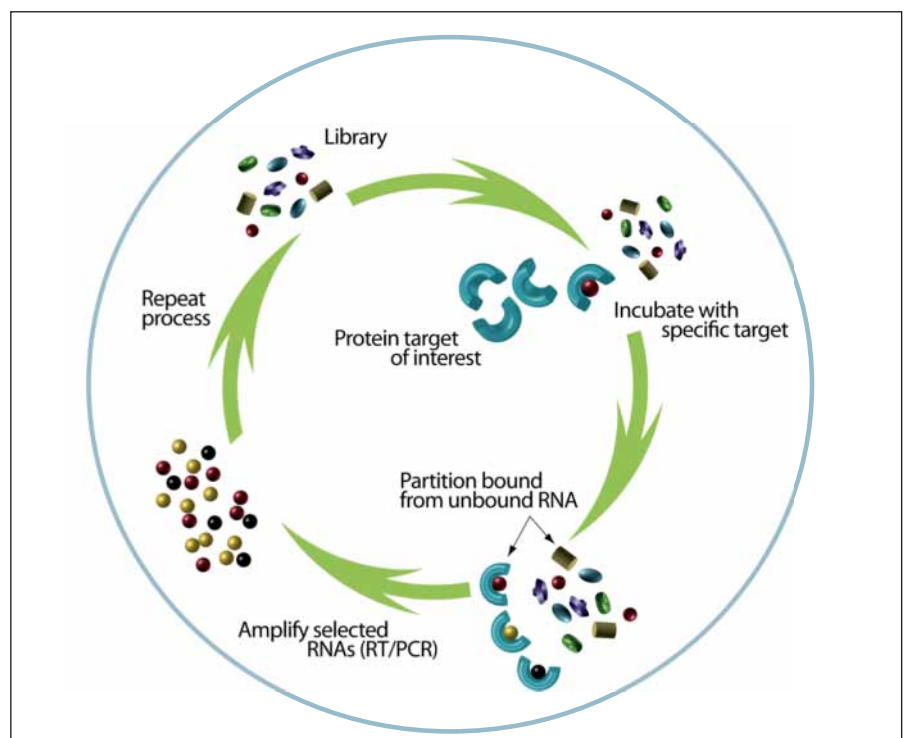
Basic science	Clinical science
Aptamers generated using SELEX (1)	Phase 1A first in human healthy volunteer study with fIX aptamer and antidote (2)
Aptamers generated against coagulant proteins (fIX, fX, fVII-TF) (3–4)	Phase 1B study in subjects with stable CAD receiving aspirin with/without clopidogrel (20)
Complementary oligonucleotides generated, that alter aptamer conformation and actively reverse anticoagulant activity (15, 16)	Phase 1C study of repeated fIX aptamer and antidote administration (21)
Aptamer generated against VWF with complementary antidote tested <i>in vitro</i> and in animal models of thrombosis (36)	Phase 2A study of patients undergoing elective PCI with fIX aptamer as sole anticoagulant and either partial or complete reversal (22)
	First in human Phase 1 PK/PD study with VWF aptamer- ARC 1779 (37)
SELEX, Systematic Evolution of Ligands by Exponential Enrichment; f, factor; TF, tissue factor; VWF, von Willebrand factor; CAD, coronary artery disease.	

Modifications of the original SELEX method have been developed and employed by basic and translational scientists. The methods include: convergent SELEX (initial rounds with a complex proteome and later rounds with a target protein); blending SELEX (library of oligonucleotides covalently bound to a ligand or protein target); mirror-image SELEX (selection of enantiomers of natural compounds); toggle SELEX (selection of several protein targets or targets of differing species through alternating rounds); cell-SELEX (selection against a specific cell type); 2D-SELEX (recognition of stem-loop structures); genomic SELEX (employment of a library based on genomic sequences), and tailored SELEX (library without fixed sequences) (6–8).

### Unique properties

The affinity of an aptamer to a target protein ranges from low picomolar to low nanomolar. The high-affinity property of aptamers is based on specific complementary contacts between functional groups on both the nucleic acid and the target protein (or small molecule). The three-dimensional arrangement of contact also assures specificity, permitting successful discrimination of the target and non-target homologous proteins (9–11).

A technique of maximum common binding modes (MCBM) has been employed to determine small molecule-protein docking (12). Patterns of interactions are modelled using ligand receptor



**Figure 1: SELEX (Systematic Evolution of Ligands by EXponential enrichment) is employed to isolate and subsequently purify high-affinity nucleic acid ligands (see text for full description).**

Aptamers	Traditional intravenous anticoagulants
Target selectivity	Complex PK
Target specificity	Complex PD
PK-PD relationship closely coupled	PK-PD relationship suboptimal
Active titration and reversal platform	Titration difficult or untested
Very low likelihood of off-target adverse effects	Narrow therapeutic index – Bleeding – Thrombosis prevention
Potential for multiple, concomitant biology/clinically based targets	Antidote or active reversal platform unavailable, poorly tested or potentially toxic
Tailored plasma half-life according to clinical indication with intravascular metabolism	Off-target adverse effects (particularly high molecular weight, polydisperse heparins) relative common

**Table 2: Comparative properties of aptamers and traditional anticoagulants.**

interaction fingerprints from known co-crystal structures, including thrombin. Simulated annealing molecular dynamics has also been employed for conformational sampling and optimisation. The technique includes structural information from both the ligand and protein active site enabling identification of the optimal conformation for ligand-protein complex formation (13).

## Aptamers as antithrombotic agents

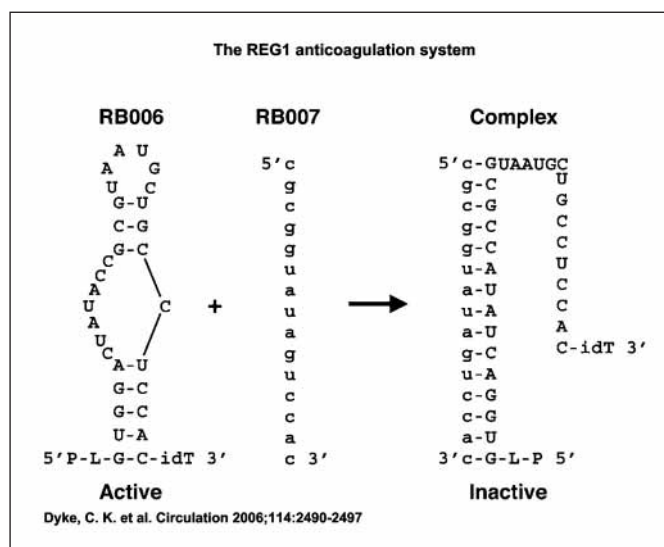
A cell-based model of coagulation (14) has provided guidance for selecting targets for aptamer development (►Table 2). In this readily translatable model, initiation of coagulation takes place on

tissue factor-bearing cells such as monocytes, macrophages and endothelial cells as well as microparticles derived from platelets. The latter may be particularly important in the pathobiology of coronary arterial thrombotic disorders. In the presence of factor (f) VIIa complexed with tissue factor, activation of fIX and fX generates a small, but sufficient amount of thrombin for platelet activation, which in turn provides a surface for coagulation protein assembly, further thrombin generation and clot growth.

### Aptamers targeting factor IXa

The REG1 Anticoagulation System™ (Regado Biosciences, Durham, NC, USA) consists of RB006, the active aptamer drug component that inhibits fIXa with high affinity and specificity, and RB007, a complementary oligonucleotide antidote (►Fig. 2) (5).

RB006 and related precursor compounds led to the development of the current generation molecule found within the REG1 Anticoagulation System™ (Regado Biosciences Inc.) that effectively inhibits fX activation *in vitro*; prolongs plasma clotting times *in vitro*; systemically anticoagulates small and large animals and non-human primates following an intravenous bolus injection; reduces bleeding in small animal models with administration of the complementary reversal agent; prevents arterial thrombosis in several small animal models of vascular injury; replaces unfractionated heparin (UFH) in a miniswine model of cardiopulmonary bypass; and attenuates the systemic inflammatory response provoked by extracorporeal circulation more effectively than UFH (15–16, 17).



**Figure 2: The REG1 anticoagulation system® is composed of the drug RB006 and the oligonucleotide antidote to RB006 (RB007), which binds to RB006 via Watson-Crick base pairing and thereby neutralises its pharmacological effect.** Antidote RB007 and the positions within RB006 to which it pairs are highlighted in red. P indicates polyethylene glycol; idT, inverted deoxythymidine. (modified from Dyke, *Circulation* 2006; 114: 2490–2497).

### *In vitro* coagulation and thrombin generation assessment

The effect of RB006 on thrombin generation and fibrin formation has been studied in healthy volunteers using a calibrated automated thrombogram (CAT), thromboelastography (TEG) and thromboelastometry (ROTEM) (18). Measurements were performed in decalcified whole blood (TEG, ROTEM) or plasma (CAT) samples at increasing RB006 concentrations of 6, 12 and

24 µg/ml. RB006 prolonged the lag time (rate), decreased the peak of thrombin generation and delayed thrombin-mediated clot formation in a concentration-dependent manner (► Fig. 3) (5, 18).

### Metabolism

RB006, is a modified ribonucleic acid (RNA) aptamer, 31 nucleotides in length and stabilised against endonuclease degradation by the presence of 2' fluoro and 2'-O-methyl sugar-containing residues, and stabilised against exonuclease degradation by a 3' inverted to a deoxythymidine cap. Clearance of free RB006 involves both intravascular and to a lesser degree renal mechanisms. The latter route is predominantly operative in clearance of a biologically inactive component of a proteolysed parent molecule. RB007 is a 2'-O-methyl RNA oligonucleotide 15 nucleotides in length. The 2'-O-methyl modification confers moderate nuclease resistance. RB007 is cleared rapidly from the circulation. The RB006-RB007 complex is both stable and biologically inactive. Its clearance is believed to involve metabolism of the active agents to inactive nucleotides by endogenous endonucleases. Given the rapid clearance of RB007 and the RB006-RB007 complex, re-anticoagulation can be achieved within minutes (min) of RB007 administration by administering RB006.

The durability of aptamer neutralisation is consistent with the predicted thermodynamics of the nucleotide double helix formation which entails base pairing via multiple high energy hydrogen bonds. In addition, given the differing half-lives of the antidote (minutes) and the irreversible binding of antidote to the active aptamer, re-administration of the active aptamer is capable of immediately restoring a state of anticoagulation, permitting a clinician to respond appropriately to existing clinical conditions. The same is potentially true for aptamers serving as platelet (or their major ligand) antagonists.

## Clinical development program

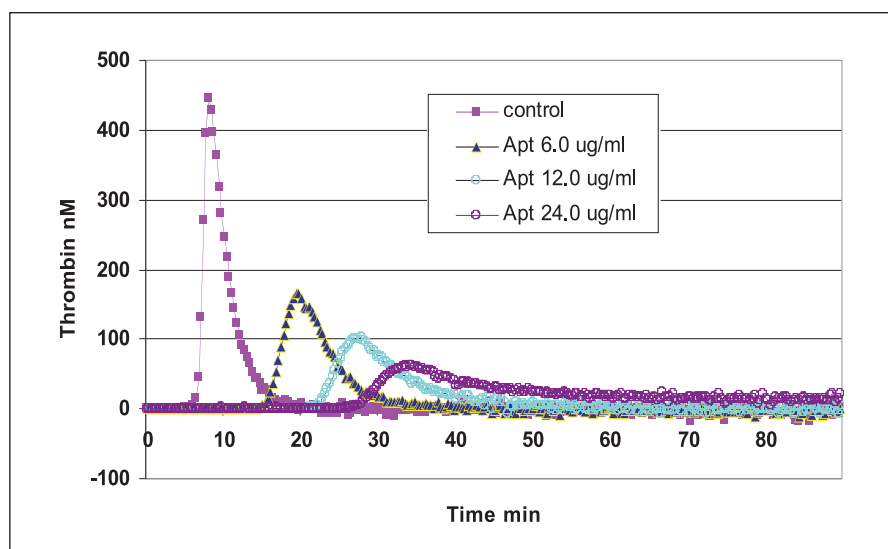
### Regado phase 1A

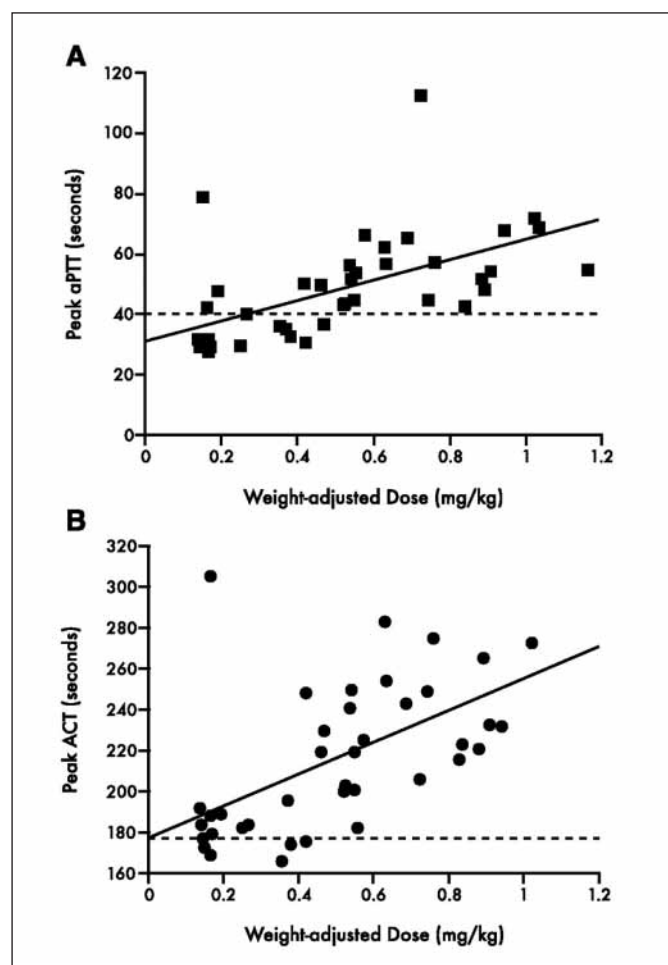
Regado 1A was a phase 1, first-in-human study of an aptamer-based inhibitor to factor IXa (RB006) and its complementary control agent (RB007). A total of 85 healthy volunteers older than 18 years of age (median age 32 years) participated in a subject-blinded, dose escalation, placebo-controlled study and received a bolus of RB006 or placebo followed 3 h later by a bolus of RB007 or placebo (19). The doses of RB006 – 15 mg, 30 mg, 60 mg and 90 mg were selected to provide a large margin of subject safety, and to parallel the pharmacodynamic effects, based on the activated partial thromboplastin time (APTT), and an *in vitro* dose-response curve. The fixed dose of RB007 was twice the RB006 dose chosen (antidote-to-drug ratio of 2:1-representing a 10-fold molar excess), and represented a four-fold increase above the minimal amount required *in vitro* to fully neutralise RB006 and return fIX activity to its baseline level.

In subjects receiving RB006, the APTT increased rapidly and dose-dependently, with a consistent pharmacodynamic effect over the initial 3 h. The duration of effect was also dose dependent, with a return to baseline APTT at 3 h, 20–24 h and 30 h, respectively, for the 15, 30 and 60 mg doses of RB006. Prolongation of the prothrombin time (PT), as predicted from pre-clinical experiments, was not observed at any dose of RB006.

To determine the relationship between dose of RB006, APTT values (obtained 15 min after drug administration) and fIX inhibition, the relative increase in APTT was compared with the fIX activity assay calibration curve. There was a 1.1-, 1.3-, 2.1- and 2.9-fold increase compared to baseline in APTT, corresponding to a 35%-40%, 80%, 98% and >99% loss of fIX activity, respectively (19). Activated clotting time (ACT) values followed a similar dose-response pattern, with relative increases of 1.1-, 1.3-, 1.4- and 1.5-fold, respectively. A clear relationship between RB006 dose ad-

**Figure 3: Effect of increasing concentrations of anti-IXa aptamer on thrombin generation with diluted actin as an activator.** A) The peak of thrombin generation is progressively decreased and lag time is increased in a concentration-dependent manner. For better visual display some of the data points were removed. (modified from Tanaka. *Thromb Haemost* 2009; 101: 827–833.





**Figure 4: Relationship between aPTT (A) and ACT (B) achieved and weight-adjusted dose of RB006.** Horizontal dashed line denotes upper limit of normal for the assays. Maximal aPTT and ACT levels (seconds) achieved were correlated with RB006 dose divided by body weight (kg). All subjects receiving active drug ( $n=41$ ) were included.  $r=0.70$ ,  $p<0.001$  for aPTT;  $r=0.67$ ,  $p<0.001$  for ACT. (modified from Dyke, *Circulation* 2006; 114: 2490–2497).

justed for weight and pharmacodynamic effect was observed (correlation coefficient, 0.75;  $p<0.001$ ).

Administration of RB007 resulted in a rapid and durable return of the APTT to baseline values. Complete neutralisation occurred, on average, within 1–5 min of drug administration (19).

Overall, RB006 and RB007 were well tolerated, with minimal bleeding at intravenous line sites. Serial measures of complement Bb did not reveal evidence of complement activation (as observed with first generation oligonucleotide siRNA with differing chemistries). One serious event, an episode of transient encephalopathy was reported.

### Regado phase 1B

The Regado phase 1B study (20) randomised 50 subjects between the ages of 50 and 75 years with stable coronary artery disease on

aspirin and/or clopidogrel to an intravenous bolus of RB006 (15, 30, 50 or 75 mg) alone, or RB006 followed by RB007, given 3 h later, at doses of 30, 60, 100 and 150 mg, respectively. As in Regado phase 1A, RB006 and RB007 were well tolerated, with no major bleeding, serological evidence of complement activation or other serious adverse events during the seven-day follow-up period. There were five subjects with minor bleeding at peripheral intravenous line sites. Among subjects who did not undergo reversal with RB007, three developed access site haematomas. Bleeding was not dose-dependent. The overall incidence of bleeding events did not differ for subjects receiving both aspirin and clopidogrel compared with those receiving single antiplatelet therapy.

There was a dose-dependent increase of APTT and ACT values 10 min after RB006 administration. Reversal of anticoagulant effects with RB007 was rapid, within 1–2 min of administration, predictable, consistent and durable over the pre-specified 3 h duration (20). There was no evidence of rebound anticoagulation that would suggest a dissociation of the RB006–RB007 complex. A residual anticoagulant effect at 12 h was evident among subjects receiving RB006 at the 50 mg and 75 mg doses followed by placebo RB007.

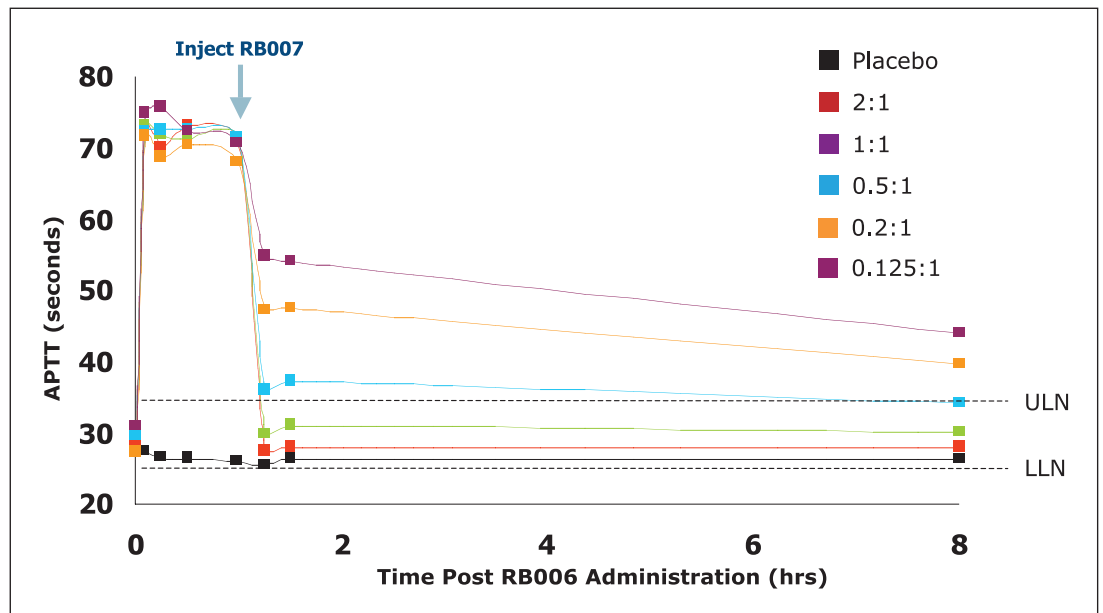
Pharmacokinetics and pharmacodynamic measures, to include RB006 plasma concentrations, fIXa activity and APTT values, derived from the phase 1A and 1B studies provided the required information to elucidate RB006 dose-response relationships according to subject body weight—an important observation for subsequent dose selection in phase 2A (► Fig. 4) (20).

### Regado phase 1C

Regado 1C was a double-blind, single centre study that included 39 healthy volunteers  $\geq 21$  years of age randomised sequentially to either a weight-adjusted dose of RB006 ( $0.75 \text{ mg kg}^{-1}$ ), followed by varying, de-escalating doses of RB007, or double placebo (21). On treatment days 1, 3 and 5, subjects received RB006 (repeat drug dosing) and RB007 (repeat active control agent dosing) 60 min later, ranging from a 2:1 to 0.125:1 antidote/drug ratio ( $1.5\text{--}0.094 \text{ mg kg}^{-1}$ ) or placebo. Blood samples for APTT, PT and complement Bb, as well as renal and liver function tests were collected daily for six days and again on a follow-up visit at day 10–14. In addition, whole blood ACT and APTT measurements were made using whole blood point-of-care coagulation monitors.

Highly reproducible APTT measurements were observed with each of the three drug-control agent cycles. Overall, the relative effect of RB006 on APTT values was a 2.5-fold increase from baseline. There was low inter-subject variability (coefficient of variation 5.5%). RB007 restored APTT levels to baseline (determined prior to RB006 administration) with all consecutive drug-control cycles. Descending doses of RB007 ( $<2:1$  weight to weight control agent/drug ratio) achieved partial neutralisation of RB006, with dose ratios of 0.5:1, 0.3:1, 0.2:1 and 0.125:1 causing a reversal of approximately 84%, 74%, 51% and 41% of the RB006 anticoagulant activity, respectively, within 15 min of administration (► Fig. 5). (21). Plasma levels of fIX activity, while generally lower on day 6 of follow-up than at baseline, were still well within normal limits (60–150%). There was a curvilinear relationship between labora-

**Figure 5: Recovery of plasma activated partial thromboplastin time (APTT) with differing doses of RB007.** The dashed horizontal line denotes the upper limit of normal for the aPTT assay. Mean  $\pm$  SD APTT values are displayed (modified from Chan. *Circulation* 2008; 6: 789–796.)



tory plasma APTT and point-of-care whole blood APTT, with a correlation coefficient of 0.76. There were no major bleeding or serious adverse events in subjects receiving RB006. There were six minor bleeding events. Mean complement Bb levels remained within pre-defined limits throughout the study period. The descending control agent dose strategy studied in Regado 1C was instrumental in selecting the drug reversal methodology for the phase 2A pilot study.

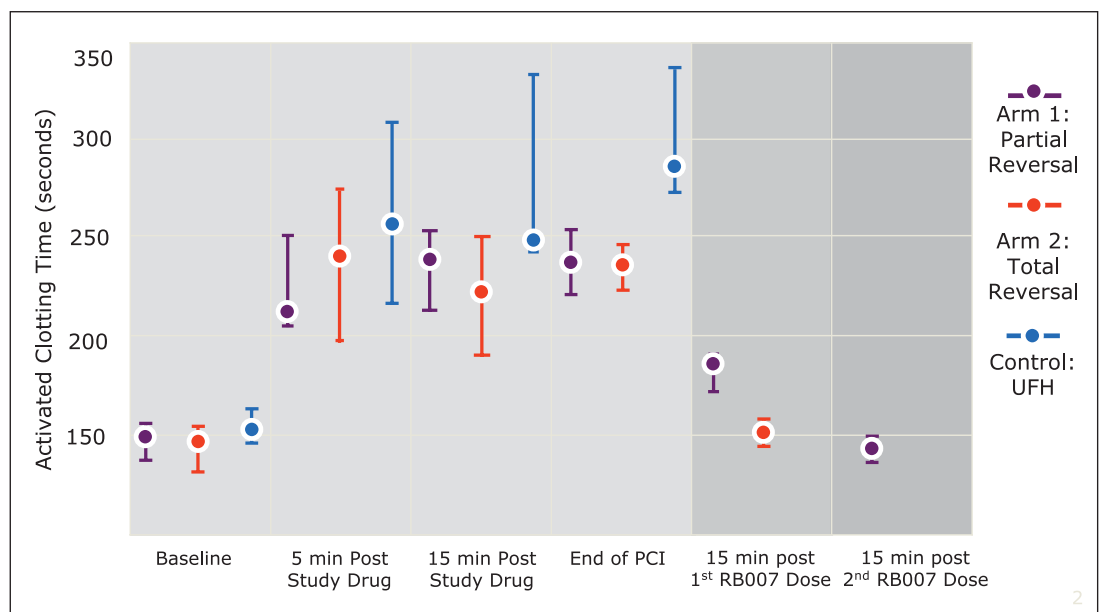
**Regado phase 2A**

The encouraging experience in the phase 1A, 1B, and 1C studies with the anti-f<sub>IXa</sub> aptamer (RB006) and control agent (RB007),

led to a phase 2A pilot study, called Reversal-PCI (percutaneous coronary intervention)( NCT00113997), to further assess the feasibility and safety of its use in low-risk patients undergoing PCI (22). The dose of 1 mg/kg RB006 was chosen to inhibit f<sub>IX</sub> activity by >99% (22). All patients received aspirin and a loading dose of clopidogrel before PCI.

The primary endpoints of Reversal-PCI were major bleeding (in hospital) and the composite of death, non-fatal myocardial infarction (MI) and urgent target vessel revascularisation through day 14. A prospective and adjudicated assessment of catheter, equipment and periprocedural thrombosis was included as a secondary endpoint. Reversal-PCI represented the first clinical use of the REG1 system, therefore the study design included a roll-in

**Figure 6: Activated clotting time (ACT) measurements in subjects participating in the REVERSAL-PCI Study.** ACT levels exceeded 200 seconds after a 1 mg/kg intravenous dose of RB006. The values decreased toward baseline following either partial or complete reversal with RB007. (modified from Cohen. *ESC* 2009).



Study Arms	Assigned Interventions
REG1-a: Experimental fixed dose of RB006 (anticoagulant) variable blinded dose of RB007 (control agent)	Drug: REG1 IV bolus dose. May be re-dosed. Control agent given as single re-dose at completion of intervention
REG1-b: Experimental fixed dose of RB006 (anticoagulant) variable blinded dose of RB007 (control agent)	Drug: REG1 IV bolus dose. May be re-dosed. Control agent given as single re-dose at completion of intervention
REG1-c: Experimental fixed dose of RB006 (anticoagulant) variable blinded dose of RB007 (control agent)	Drug: REG1 IV bolus dose. May be re-dosed. Control agent given as single re-dose at completion of intervention
REG1-d: Experimental fixed dose of RB006 (anticoagulant) ' variable blinded dose of RB007 (control agent)	Drug: REG1 IV bolus dose. May be re-dosed. Control agent given as single re-dose at completion of intervention
Heparin: Active comparator Heparin per standard of care at the local institution	Drug: REG1 IV dose per standard of care at the local institution

Table 3: Regado phase 2B trial – RADAR.

phase with two patients treated with RB006 and an intravenous glycoprotein (GP) IIb/IIIa antagonist to ensure effective platelet inhibition. After safety committee review, 24 patients were randomised in a 5:1 fashion to either the REG1 Anticoagulation system or unfractionated heparin (UFH). Two post-procedure drug reversal strategies were investigated – partial reversal following successful PCI, followed 2 h later by complete reversal and arterial sheath removal; or complete reversal following successful PCI with immediate arterial sheath removal. Pharmacodynamic response to RB006 was assessed with ACT and whole blood APTT using a point-of-care device. At 5 min after intravenous RB006 administration and at completion of PCI, whole blood APTT levels were 148.5 and 145 seconds (s), respectively, and ACT levels were 223 and 236 s, respectively. Partial reversal with RB007, administered in a ratio of 0.2:1 with respect to RB006, resulted in  $51 \pm 14\%$  to  $68 \pm 15\%$  reversal of RB006 activity, while complete reversal with RB007 given at a ratio of 2:1 with respect to RB006 resulted in  $93 \pm 11\%$  to  $103 \pm 13\%$  reversal of RB006 activity (► Fig. 6).

### Regado phase 2B

The RADAR (Study assessing the REG1 Anticoagulation System compared to Heparin in subjects with Acute Coronary Syndrome) Study (NCT00932100) is an ongoing randomised, partially-blinded, international, multi-centre, active-controlled, dose-ranging phase 2B investigation designed to assess the safety, efficacy and pharmacodynamic properties of the REG1 Anticoagulation System™. A total of 800 patients with acute coronary syndrome (ACS) undergoing cardiac catheterisation with intent for PCI will be enrolled, randomly assigned to one of four REG1 treatment arms utilising a 1 mg/kg dose of RB006 (selected to achieve >99% inhibition of fIXa activity) and one of four RB007 dosing strategies after cardiac catheterisation designed to reverse 25%, 50%, 75%, or 100% of RB006-induced anticoagulant activity. Patients in these groups will be compared to a control arm randomised to heparin (unfractionated or low molecular weight) (► Table 3) and followed to assess the composite of major or minor bleeding at 30 days (primary outcome measure).

### Aptamers targeting factor IIa (thrombin)

Bock et al. (23) developed single-stranded DNA aptamers to thrombin with binding affinities in the range of 25 to 200  $\mu\text{M}$ . Several of the 32 aptamers selected from a DNA pool inhibited thrombin-catalysed conversion of fibrinogen to fibrin by up to 50% with plasma concentrations of 25  $\mu\text{M}$ .

Using a single-stranded (SS) DNA oligonucleotide isolated initially by Bock (GGTTGGTGTGGTTGG), Griffin et al. produced a novel nucleotide-based thrombin inhibitor (SS DNA molecule) and a scrambled control using solid-phase phosphoramidite chemistry. They then conducted a series of studies in cynomolgus monkeys to determine the *in vivo*-anticoagulant properties (24). Given the molecule's short circulating half-life (approximately 108 s in animals), a continuous intravenous infusion was required to maintain adequate plasma concentrations. The plasma PT was prolonged in a dose-dependent manner, with a plateau effect 10 min after drug initiation. Thrombin-induced platelet aggregation was also inhibited by the SS DNA aptamer. Platelet aggregation in response to collagen stimulation was not affected, supporting thrombin's specific interaction with protease activated receptor 1 (PAR-1).

An infusion of the thrombin aptamer was administered proximal to a haemofiltration unit in anaesthetised male sheep. There was significant prolongation of the PT, approximately two-fold in the haemofiltration circuit, whereas the systemic clotting time was essentially unchanged. In an *in vitro*-system (human platelet-rich plasma), the aptamer (0.07–1.45  $\mu\text{M}$ ) exhibited dose-dependent inhibition of thrombin-induced platelet aggregation. The concentration of aptamer required to achieve 50% inhibition ( $\text{IC}_{50}$ ) of platelet aggregation, as determined by planimetry of the aggregation tracing, was approximately 70 to 80 nM.

The thrombin aptamer was compared with UFH in a model of clot-bound thrombin. At a concentration of 3  $\mu\text{M}$ , fibrinopeptide A level, a marker of thrombin activity, was reduced by 80%, whereas UFH had no discernable effect. Applying an *ex vivo* whole-artery angioplasty model in rabbits, the investigators were able to document the aptamer's (2  $\mu\text{M}$ ) ability to reduce platelet deposition at

both low and high shear rates and suppress thrombin activity even in the presence of high concentrations of UFH (2 U/ml) (25).

The thrombin aptamer was subsequently tested in a canine cardiopulmonary bypass model to determine its anticoagulant efficacy, pharmacodynamics and pharmacokinetics. At a continuous infusion rate of 0.5 mg/kg/min, the ACT increased from a baseline value of  $106 \pm 12$  s to  $187 \pm 8$  s, with a further increase during haemodilution ( $259 \pm 41$  s) and during cardiopulmonary bypass ( $>1,500$  s). The observed increase in ACT closely paralleled plasma concentrations as determined by high-performance liquid chromatography. The calculated plasma elimination half-life was 1.9 min at baseline, increasing 3.5- to 4.0-fold during bypass, suggesting either a role for the pulmonary vasculature in aptamer clearance, altered renal clearance or non-specific endocytosis. The ACT returned to baseline within 5 min of drug discontinuation. Post-operative bleeding (chest tube drainage) was considered minimal (95 to 150 ml) over a 2 h observation period (26).

DNA aptamers that bind distinct epitopes on the thrombin molecule have also been developed over the past decade. Unlike earlier compounds that bound the fibrinogen-recognition (anion-binding) exosite, a 29-nucleotide single-stranded DNA ligand to human thrombin with a  $K_d$  of approximately 0.5  $\mu$ M inhibited thrombin-catalysed fibrin clot formation *in vitro* through binding of the heparin-associated exosite (27).

Although the optimal site for thrombin inhibition has not been established, aptamers based on the 15-nucleotide consensus sequence that bind the anion-binding exosite-I reduce heparin cofactor II-mediated thrombin neutralisation (with or without heparin), but have no effect on antithrombin-III activity (28).

Among several architectures or structural arrangements that account for an aptamers binding selectivity and affinity, the G-quadruplex (G-4) structure shows inhibitory properties against thrombin (29). The folding of aptamers into intricate conformations is a characteristic that offers considerable plasticity and diversity (30).

A thrombin aptamer, Nu172 (Nuvelo™, San Carlos, CA, USA) underwent phase 1 testing in 24 healthy male volunteers who received a 2 mg/kg bolus, followed by an escalating infusion for 4 h. In all subject cohorts, NU172 produced a dose-dependent increase of ACT, PT and APTT. All coagulation measurements remained stable during the continuous intravenous infusion. A phase 2 clinical trial (SNAP-CABG-OFF) (NCT00808964) is planned to evaluate 30 subjects undergoing primary, elective, off-pump coronary bypass grafting.

## Bivalent thrombin aptamers

A bivalent aptamer that binds to thrombin with high affinity and occupies both anion binding exosites has been developed by Müller et al. (31). HD1–22 prolonged the thrombin time, APTT, ecarin clotting time and lag time of a tissue factor-triggered thrombin generation assay in a dose-dependent manner. The anticoagulant activities were fully reversed with an antidote oligodeoxynucleotide (1, 18, 31).

## Aptamers targeting platelet functional ligands

### Von Willebrand factor aptamers

Glycoprotein (GP) Ib-IX-V is a platelet adhesion receptor belonging to a leucine-rich family of proteins. Its major function is to initiate platelet adhesion at high-shear stress, facilitating aggregation and thrombus formation (32). Following von Willebrand factor (VWF) binding to GP Ib-IX-V, platelets are activated, undergo cytoskeletal shape change and secrete proteins, such as thromboxane A2 that recruit additional platelets to the developing thrombus (32). VWF-bound GP Ib-IX-V induces a conformational change in the GP IIb/IIIa receptor, transforming it from an inactive low-affinity state to an active receptor that binds additional VWF or fibrinogen with high affinity. Thus, VWF plays a pivotal role in platelet adhesion, activation and aggregation *in vivo*. Along with GP IIb/IIIa, GP Ib-IX-V is the only platelet receptor that has a non-redundant role in haemostasis and thrombosis (32–35). Thus, inhibitors of the VWF-GP Ib-IX-V interaction may represent an attractive approach to antithrombotic therapy.

Laboratory-based *in vitro*-evaluation experimentally supports the hypothesis that a targeted oligonucleotide aptamer against VWF would inhibit platelet adhesion in PFA-100 platelet shear force assays and ristocetin-induced platelet aggregation assays (36). Utilising a convergent SELEX approach, several oligonucleotides with molecular weights ranging from 8 to 15 kDa were identified that bound to VWF with high affinity (dissociation constants ( $K_d$ ) less than 20 nM). Furthermore, rational design of complementary oligonucleotides functioned effectively as active reversal agents, abolishing *in vitro*-inhibition of platelet adhesion within 2 min and in a sustained manner for up to 4 h following administration (36).

*In vitro*-selection, aptamer stabilisation and conjugation to a 20 kDa PEG, generated a nuclease resistant aptamer, ARC 1779, that bound to the VWF A1-domain. ARC 1779 inhibited botrocetin-induced platelet aggregation ( $IC_{90} \sim 300$  nM) and shear force-induced platelet aggregation ( $IC_{95} \sim 400$  nM). The aptamer reduced adhesion of platelets to collagen-coated matrices, and attenuated formation of thrombin on endothelium denuded porcine arteries. Last, ARC 1779 given as a continuous intravenous infusion inhibited the formation of occlusive thrombi in a primate thrombosis model.

ARC1779 was administered in a phase 1 double-blind, placebo controlled study of 47 healthy volunteers to establish pharmacokinetic, pharmacodynamic, and safety profiles (37). There were no adverse events, excess bleeding, or deaths reported; however, one subject experienced a hypersensitivity reaction following intravenous bolus administration that subsequently prompted a change in the dosing strategy (37). A phase 2 study of this agent in patients with non-ST elevation MI undergoing PCI was initiated in October of 2007, but terminated for unknown reasons, according to the most recent information available on the Food and Drug Administration's ClinicalTrials.gov website. Similarly, a study of the

pharmacokinetics, pharmacodynamics and safety of ARC1779 in patients with type 2B von Willebrand disease (NCT00694785) was withdrawn prior to recruitment. A third study, ARC1779 in patients with von Willebrand factor-related platelet function disorders (NCT00632242) included 28 patients with either thrombotic thrombocytopenic purpura (TTP) in remission, acute TTP or type 2B von Willebrand disease. The study has been completed, but neither published nor presented data are available. Last, ARC1779 is being tested in patients undergoing carotid endarterectomy to determine its effect on cerebral microembolism (NCT00742612).

## The importance of control and active reversal in drug development and clinical practice

In principle, the activity of an antithrombotic drug could be controlled after its administration by either formulating the drug to possess a rapid off-set of activity (i.e. a short half-life), by attenuating drug therapy or replacement products (e.g. protamine, recombinant procoagulant factors), or by injecting a specific antidote or reversal agent to neutralise, potentially in a graded fashion, the activity of the drug. Of these, active control may be preferable to the other methods, each of which carry their own inherent risks and are in themselves difficult to titrate to a desired pharmacodynamic effect. In addition, the availability of an active reversal agent allows an antithrombotic drug to be formulated to possess a longer circulating half-life while still maintaining an enhanced safety profile, thus enabling the drug to be used in a wide variety of indications, including “bridging strategies”. Data obtained in the Regado phase 1A, 1B, 1C and 2A studies firmly support the construct of active reversibility for aptamers.

## Conclusion

The development of single-stranded protein-binding oligonucleotides represents a new and potentially important advance in antithrombotic therapy. Preclinical work with aptamers directed at fVIIa-tissue factor complex, fIXa, fXa and thrombin has yielded encouraging results, particularly the construct of drug-active reversal agents, which adds a clinically relevant dimension to targeted and controlled treatment regimens for patients with thrombotic disorders. The clinical development program for REG1, a fIXa aptamer, and its complementary reversal agent has been particularly robust, with an ongoing phase 2B clinical trial. Aptamers directed against other coagulation proteins and major platelet ligands are in a relatively early stage of clinical investigation. Future studies will delineate the application of aptamer technology in human diseases and provide vital mechanistic and management insights for clinical translation as well (20, 38).

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